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Investigating cell sorting and analysis of the proprietary cell-BOCS platform

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***Abstract*—This project comprises investigation of cell sorting, of both artificial beads and biological cells, and analysis of the proprietary cell-BOCS - Bio Optofluidic Cell Sorter, a table top cell sorter using optical manipulation. The analysis is more specific on broadening the ways of detection of cells and upgrading the optical manipulation system. Detection is done by bright-field imaging but there is a specific need to expand the detection criteria to other fields, for example fluorescence. Positively identified particles are sorted out by means of "optical catapulting" with a spatially modulated laser beam. The anticipated project output is a cheap, compact and easy-to-operate cell sorter that is able to detect and manipulate fluorescent microspheres, fluorescent labelled yeast cells and potentially erythrocytes in a microfluidic stream.**

***Index Terms*—Cell sorting, spatial light modulation, fluorescence**

INTRODUCTION

Flow cytometry is an intensively used tool for single cell analysis and cell sorting based on fluorescence signals in a broad range of research fields. It has however some limitations and drawbacks that stand in the way of further adaptation and proliferation (reference). There is the high cost, need for dedicated personnel, large sample volumes and the lack of miniaturisation. The solution for these problems is microflow cytometry. Microfluidics can start a revolution in chemistry and biotechnology comparable to the rise of the silicon microchip in electronics [1]. It provides a way of precisely controlling and mimicking the cellular and chemical environment. The use of disposable chips and small sample volumes (10-100 μl) makes this technique very attractive for further research. In this project, sorting based on fluorescence in a microfluidic setup is further investigated. The optical manipulation technique utilises a near infrared laser that exerts forces on particular beads or cells of interest, for example the ones that are fluorescing. The laser beam can be pointed towards the desired position by means of a binary or blazed grating and simple fourier optics. The selected particles are catapulted out of the stream and the system acts as a cell sorter or filter. The aim is to incorporate the findings from this project into the cell-BOCS - Bio Optofluidic Cell Sorter - setup developed by OptoRobotix ApS [2] [3]. In the original setup, sorting is based on cell morphology, uncluding the shape, size and color of cells [4]. The morphology is sensitive to external factors and diseases so it is a good criterion for detection. Adding a fluorescence imaging subsystem to the cell-BOCS can make the technology even more versatile and broaden the range of applications.

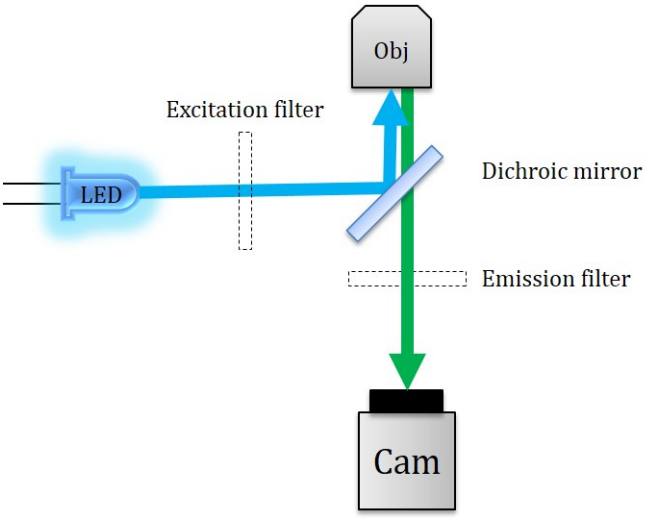

Fig. 1: Epi-fluorescence imaging system

I. FLUORESCENCE IMAGING AND CELL SORTING

A. Fluorescence imaging

Fluorescence imaging is one of the most powerful and fundamental techniques used in any life science research lab. The phenomenon underlying this imaging technique is fluorescence, discovered by Stokes in 1852. Autofluorescence as well as fluorescence using exogenous fluorophores is extensively researched for biological applications. Combination of fluorescence imaging with high-end image processing software provides new insights in cellular cytometry and dynamics. The most widely used fluorescence imaging system is based on epi-fluorescence microscopy. In order to achieve high sensitivity, resolution and signal-to-noise ratio, an optimal combination of optical components, light sources and cameras should be found. Specifications for the filters, lenses, light source and detector are highly dependent on the application and the fluorescent probes. The basic epi-fluorescence microscope is shown in figure 1.

Sensitivity and specificity of the fluorescent signal and selectivity of immunofluorescent antibodies are the key advantages of this technique. Immunofluorescence uses antibodies and antigens to attach fluorescent probes to target cells. There are two types of immunofluorescence: direct

or primary immunofluorescence and indirect or secondary immunofluorescence. Direct immunofluorescence uses one antibody that binds to an antigen that is present on the surface of the cell. A fluorophore is attached to the antibody and in this way linked to the cell of interest. In secondary or indirect fluorescence, a primary antibody binds to the target molecule in the same way as in direct immunofluorescence. A secondary antibody conjugated with a fluorophore is added in a following step and binds to the primary antibody. Because of the particular structure of antibodies, this technique provides signal amplification as more fluorophores can be linked to each individual antigen. Direct immunofluorescence requires less steps in the staining process than indirect immunofluorescence and is therefore easier and faster. Direct immunofluorescence requires however a lot more primary antibody which is really expensive. For indirect staining, several different primary antibodies recognising different antigens on the cell surface can be used to which the secondary antibody can bind. This is possible because of constant domains in the base of the Y-shaped primary antibody.

B. Cell sorting techniques

The classic techniques used in cell sorting are fluorescence activated cell sorting (FACS), optical trapping and dielectrophoresis (DEP) [5]. These techniques can all be combined with microfluidics. Microfluidics has the ability to precisely control fluid and laminar flow, use small sample quantities and doesn't require long analysis time. Cell manipulation in microfluidics opens doors to new pre-clinical diagnostic tools. Using this *in vitro* "human-on-a-chip" approach, it is possible to mimic human tissue. This could help further research human functions and could shed light on unknown processes and pathologies [5].

The cell sorting principle in this project is a "point and shoot" approach. The power of a strongly focussed infrared laser beam aims at the detected cell of interest and kicks it out of focus like a water hose. Scattering forces are responsible for the movement of the particles.

C. Spatial light modulation

Basic knowledge of fourier analysis is in fact enough to understand the optical manipulation approach used in the cell-BOCS. If light is incident on a binary grating with a certain periodicity, the light diffracts and bright spots will be visible in the fourier plane according to the diffraction orders of the grating. Changing the periodicity of the grating will result in translation of the diffraction orders and the light beam can be directed to any desired position in the fourier domain. In practice, the pattern is sent to a spatial light modulator (SLM) and the sample plane coincides with the fourier plane forming the optical manipulation system. Digital micromirror devices and other MEMS based devices can be used to spatially modulate the laser beam. Depending on the tilt of the mirror, the light is directed towards the imaging pathway or to the surroundings where it's lost. This amplitude modulation technique is therefore inefficient in power usage.

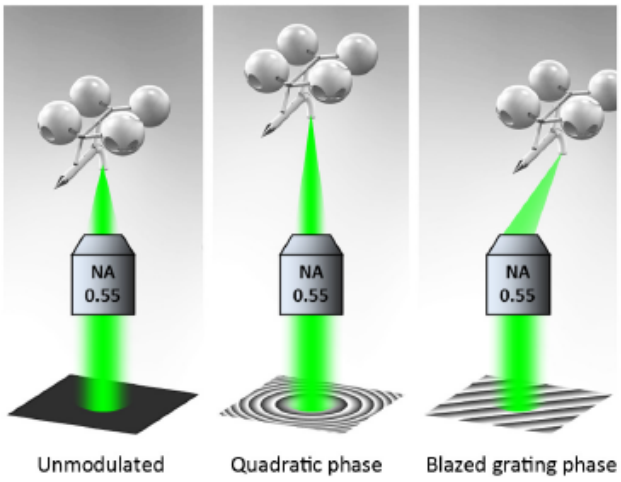

Fig. 2: Diffractive coupling to a microtool [6]

"Phase-only modulation" does not suffer from this drawback and programmable phase-only spatial light modulators are commonly used for light shaping. The LCoS technology can be used for this purpose. An LCoS is a microdisplay consisting of a silicon substrate with a liquid crystal layer on top such that it combines both the reflective and transmissive modes. Depending on the orientation of the liquid crystals, light can pass or not. Light that reaches the silicon substrate gets reflected and is modulated.

As well as binary gratings, blazed gratings are used. This type of diffraction grating was developed to achieve maximum coupling efficiency to a given diffraction order while minimising the power in the zeroth diffraction order. Over one period, the phase increases linearly between 0 and 2π and then jumps back to 0. The grating is optimised for one wavelength, the blaze wavelength. The angle of diffraction β is related to the periodicity or line spacing d according to the following formula:

$$d(sin(\alpha) + sin(\beta)) = m\lambda \tag{1}$$

where α is the angle of incidence, m the diffraction order and λ the blaze wavelength.

Figure 2 shows how phase modulation can steer beams. When there is no SLM the light is unmodulated and doesn't change direction. A blazed grating will change the position in a horizontal plane (the fourier plane). Quadratic phase changes change the vertical position (in the z direction). An objective with a low numerical aperture (NA) is used because a high NA limits the axial working distance. The relationship between phase and the lateral and axial movements is given be the following formulas based on the so called simplified blazed grating and quadratic phase approach [6]:

$$\phi_{lateral}(x, y) = \frac{2\pi}{\lambda f}(x\Delta x' + y\Delta y') \tag{2}$$

$$\phi_{axial}(x, y) = -\frac{\pi\Delta z'}{\lambda f}(x^2 + y^2) \tag{3}$$

In these equations, f is the focal distance of the objective, the unprimed coordinates x and y refer to the plane of the

SLM and the primed coordinates refer to the fourier plane where the sample is situated.

II. Setup and materials

A. The cell-BOCS

Figure 3 depicts the optical design of the updated cell-BOCS. The setup has an approximate width of 1m, depth of 50 cm and height of 50 cm. A blue LED illuminates the microfluidic chip. In this chip, there are two channels at different heights, crossing each other orthogonally. The cell sorter is able to identify particles of interest based on two criteria: cell morphology via bright-field microscopy and fluorescence emitted by conjugated fluorescent probes. The transmitted light for bright-field microscopy and the fluorescent signal are separated by a dichroic mirror with an optimal cut-off wavelength above the excitation wavelength and reaches two different cameras. Because the light emitted by the fluorescent probes has a higher wavelength than the excitation light, it gets transmitted and the rest of the light is reflected by the dichroic mirror and used to form the bright-field images. Image processing allows detection of cells that fit certain criteria and will be discussed in section III. The location of the positively identified cells coincides with the first diffraction order of a certain blazed grating and inverse fourier transformation generates the wanted pattern that can be sent to a programmable spatial light modulator. An infrared laser beam (1070 nm) is spatially modulated by the LCoS upon reflection and shoots the detected particles from the lower to the upper channel. Figure 4 shows this sorting principle. The filters are chosen such that they fit the excitation and emission spectra of fluorescein, a widely used fluorescent dye, and its derivatives. An additional infrared filter is inserted in the optical path to the fluorescence camera to remove the laser light as its intensity is much higher than the fluorescent signal and it would disturb the detection mechanism.

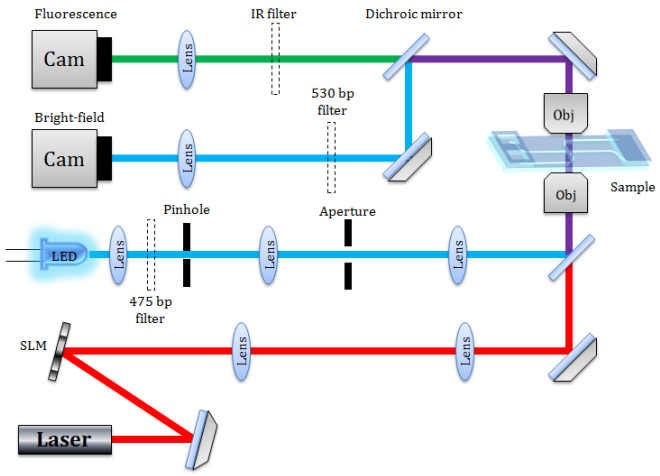

Fig. 3: Optical design of the cell-BOCS.

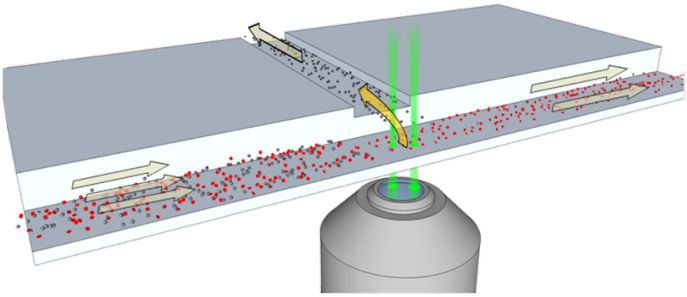

Fig. 4: Catapulting of positively identified particles in a microfluidic chip [7].

B. Fluorescent microspheres and biological cells

Unwanted effects and phenomena such as clogging and sticking to the walls of the microfluidic chip rise when working with living cells. Artificial cells or beads are in general much easier to work with because they are simply not alive. One of the biggest problems is the formation of biofilms. A biofilm is a group of microorganisms in which cells cluster together and appear when bacteria adhere to surfaces in a aqueous environment. In this project fluorescent microspheres, yeast cells and red blood cells are used to assess the performance of the cell sorter.

a) Fluorescent microspheres: Fluoresbrite Yellow-Green (YG) Reference Microspheres with a diameter of 3 μm have an excitation maximum at 441 nm and emission maximum at 486 nm, matching the fluorescein filter settings. These beads are extensively used in flow cytometry and diagnostics and have the brightest fluorescence signal in this wavelength range. The microspheres are packaged as 2.5% aqueous suspension with 1.68 $\times 10^9$ particles per ml. An ideal sample is prepared by adding 50 μl of the beads to 1 ml of ultra pure water resulting in a concentration of around 2 million microparticles per milliliter.

b) Fluorescent labelled yeast cells: Baker's yeast, budding yeast or Saccharomyces cerevisiae is a species of yeast that is widely used in laboratories for biological research. The ideal size for kicking the cells with the infrared laser light is around 10 μm. A typical cell culture is prepared by adding 0.1 ml of yeast (or around 0.1 g based on a density of 1.1 g/ml [8]) to 50 ml of ultra pure water and letting them grow overnight with the addition of some sugar. The yeast cells are stained with a fluorescein derivative Alexa Fluor 488 from Thermo Fisher [9] through a primary immunofluorescence staining protocol [10]. The Alexa Fluor 488 fluorescent probe is linked to Concavanalin A which is a lectin or carbohydrate-binding protein, originally derived from the jack-bean, that binds specifically to sugary functional groups. The glycoproteins present on the yeast cell surface are ideal binding sites for the dye.

c) Fluorescent labelled red blood cells: The blood is suspended in a mixture of NaCl and heparin. The addition of heparin, an anticoagulant, prevents clotting and makes the bloods easier to handle. The concentration of salt in blood is 0.9% so the 0.9% NaCl solution maintains the osmotic balance of salt. A typical blood sample consists of 0.2% blood, 1% heparin solution and 98.8% NaCl solution. The samples can be stored up two one week in the fridge but the cell morphology will change overtime. This is beneficial for cell sorting experiments based on the size and shape of the red blood cells.

A well-suited candidate for immunostaining of red blood cells is the Glycophorin A antibody. The human Glycophorin A protein is encoded by the GYPA gene. Glycophorins A are sialoglycoproteins, a combination of a sialic acid and a glycoprotein, present on the erythrocyte membrane. The presence of these proteins, around 1 million per erythrocyte, provides the red blood cell membrane a mucin-like appearance [11]. The erythrocytes are stained with the CD235a/Glycophorin A Antibody, FITC conjugate from Thermo Fisher. FITC is another derivative of fluorescein so again the same filter set can be used.

III. Experiments and results

A. Detection and kicking of fluorescent microspheres

Image processing generates a grayscale version of the fluorescent image. A threshold value can be set between 0 and 255 gray levels, resulting in a binary image. Figure 5 shows the detection and kicking of a fluorescent microsphere in both fluorescent and binary images. The beads are visible as white spots to which an elliptical shape can be fitted. For bright-field imaging, the ellipse is fitted to the outline of the particle of interest. Generating a grating pattern based on the position of the center of the ellipse through inverse fourier transformation and sending it to the SLM enables the infrared laser beam to kick the detected fluorescent microsphere.

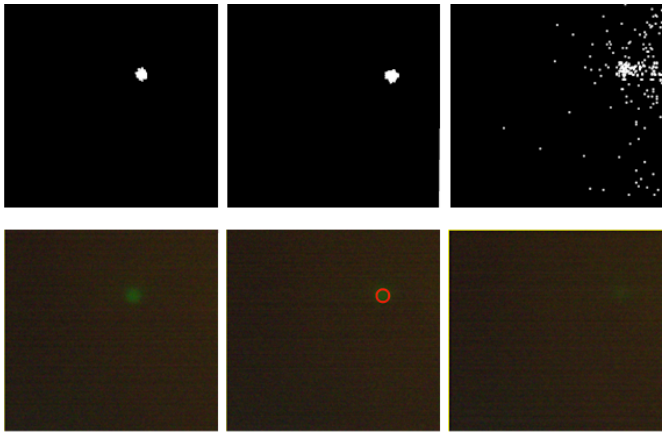

Fig. 5: Detection and kicking of a fluorescent microsphere. Top row: binary images before, at and after detection (threshold at 150). Bottom row: fluorescent images before, at and after detection (gain = 10 dB and exposure time τ = 10 ms).

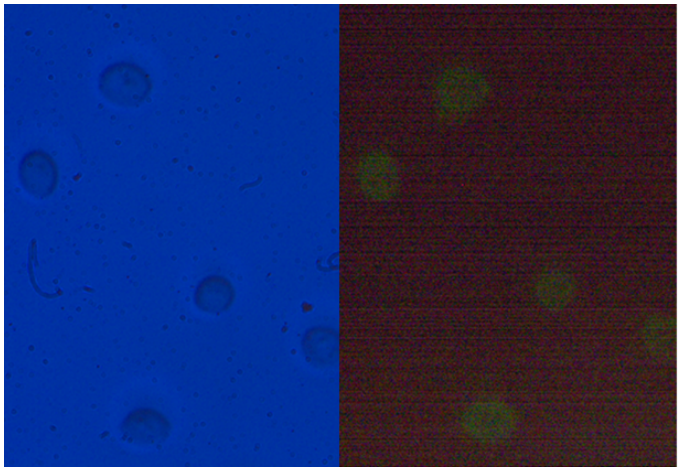

Fig. 6: Yeast cells on a microscope slide. Left: Bright-field imaging. Right: Fluorescent image (gain 15 = dB and exposure time τ = 30 ms).

B. Efficient power usage of the "shoot and point" approach

The provisional SLM was a DMD device consisting of arrays of micromirrors that can be put in two positions: reflecting the light in the direction of the optical pathway or directing the beam to the surrounding medium. The DMD serves as an amplitude modulator and this is not the optimal way to modulate light. Only 25% of the light will be directed along the optical axis in the 'on' state because of scattering. Comparing the results for the DMD device and the new LCoS microdisplay gives an idea to what extent the setup benefits from the latter in terms of power usage. Fluorescent beads are injected in the microfluidic chip which is rinsed with ethanol to prevent sticking. Once the microspheres are stationary, the chip is automatically translated at 25 μm/s to mimick a microflow. A power of minimum 1 watt is needed to effectively catapult the microspheres out of focus with the DMD, while the experiments with the LCoS only require 0.1 watt. This tenfold reduction confirms that the LCoS provides efficient power usage.

C. Sorting of fluorescent labelled yeast cells

A real microflow is set up using a pump system for real cell sorting experiments. The fluorescent signal is much lower for the yeast cells compared to the fluorescent microsperes so the gain and exposure time of the fluorescent camera have to be increased. Figure 6 shows both bright-field and fluorescent images for the labelled yeast cells on a microscope slide. For microflow experiments the exposure time has to be increased to 131 ms to ensure detection.

Figure 7 shows the detection and binary images of two flowing fluorescent labelled yeast cells. The flow can be controlled through software and the speed is set at 0.25 μl/min. A minimum laser power of 1 watt is needed to effectively kick the detected cells moving at the intersection to the upper channel.

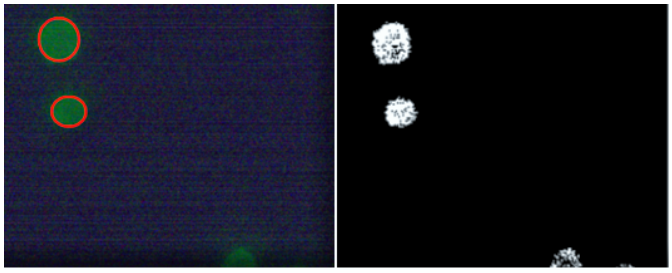

Fig. 7: Detection of yeast cells based stainend with Alexa Fluor 488 conjugated Conc A based on fluorescent imaging (gain = 15 dB and exposure time τ = 131 ms). Corresponding binary image with a grayscale threshold of 150 on the right.

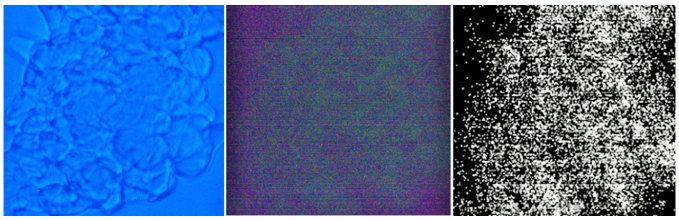

Fig. 8: Bright-field (left), fluorescent (center, gain = 15 dB and exposure time τ = 2 s) and binary image after imposing a threshold (right) of a cluster of red blood cells stained with FITC.

D. Experiments with fluorescent labelled red blood cells

Sorting blood samples can shed light on diseases such as rare anaemia, a blood condition causing a decrease of red blood cells in the blood, and circulating tumor cells in the blood stream. To assure easy handling of the blood cells, the blood samples have to anticoagulated. Primary immunofluorescence is used to stain the cell surface of the erythrocytes.

A lot of problems arose regarding the cell staining procedure. The primary antibodies are conjugated with FITC, which has a lower fluorescence intensity and is more prone to photobleaching than other fluorescein derivatives such as Alexa Fluor 488. The exposure time of the fluorescent camera is increased up to 2 seconds which is too long for cell sorting. Even with this exposure time, no fluorescent signal from individual red blood cells can be captured. The staining process causes unwanted changes to the blood like clotting and disturbance of the osmotic balance of salt. Only big groups of red blood cells emit enough fluorescent signal to be detected. Figure 8 shows a bright-field, fluorescent and binary image of such a collection of closely packed red blood cells.

Small adjustments to the staining procedures do not solve these problems. Varying the incubation time between 10 and 60 minutes, the amount of antibodies between 20 μl and 40 μl per ml of anticoagulated blood sample and all possible combinations do not have any effect. In another attempt, the Conc A antibodies for yeast cell staining are used although red blood cells only stain weakly with Conc A. Again, the fluorescent signal is too weak to be captured by the camera. A more sensitive camera should be integrated in the setup in order to be able to carry out sorting experiments with blood samples. This implies a lot of changes to the detection software and due to limited time this has not yet been done.

IV. Conclusion

The outcome of this project is a table-top cell sorter where the cells can be separated based on two criteria: cell morphology through bright-field imaging and fluorescence through fluorescent imaging. Sorting is realised by spatial light modulation of an infrared laser beam and power transfer of the laser light to the detected cells or beads. The fluorescent subsystem is important as it offers more versatility and a way to control the cell sorting. If cells of interest - for example cells with sickle cell anaemia - are stained and mixed with normal red blood cells, the performance of the cell sorter can be assessed by looking at the fluorescent signal of the sorted and unsorted cells. This was however beyond the scope of this project. The The electromagnetic force transfer was optimised by using a LCoS which resulted in a tenfold decrease in power needed to kick the detected cells. The fluorescent subsystem was built and optimised for fluorescent light around 500-530 nm because the emission spectra of most of the frequently used dyes in biology is centered around these values. These dyes include AlexaFluor 488, FITC and other fluorescein derivatives. Sorting of fluorescent labelled yeast cells was successful with 1 watt of laser power. Sorting of red blood cells based on fluorescence has not been successful so far. The use of a more sensitive fluorescent camera can possibly solve the encounter problems.

The next step in the further development of the cell-BOCS is to acquire more knowledge of biology and microfluidics within OptoRobotix ApS. There were a lot of biology-related problems such as the formation of biofilms in the microfluidic chip. Setting up the microflow with the pump system is time-consuming and has to be further facilitated. Once these problems are solved and if the cell-BOCS can be miniaturised to the size of an inkjet printer for example, it has great potential to be commercialised.

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